

LOCOMOTOR AND LIGHT RESPONSES OF LARVAE OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* (L.)

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The American horseshoe crab, *Limulus polyphemus* is an abundant and conspicuous member of the estuarine fauna of the Atlantic coast of the United States and the Gulf of Mexico. Its importance as a laboratory animal for neurophysiological, biochemical and medical research is well established (Wolbarsht and Yeandle, 1967; Cohen, 1979). Despite these facts, remarkably little has been recorded on the ecology and natural history of the species. The limited information available (Shuster, 1958; Sokoloff, 1978; Rudloe, 1979; Rudloe, 1978; Rudloe and Herrnkind, 1976) has primarily been concerned with the adult phase of the life cycle, especially the emergence of breeding crabs onto sandy beaches.

The juvenile and larval phases of the life cycle are of equal interest, however. Juveniles are important predators of the sandy intertidal community in areas adjacent to adult breeding beaches (Green and Hobson, 1970; Rudloe, 1978), while the resemblance of the larval instar to certain trilobites has long been a source of comment and is sometimes cited as an indication of the phylogenetic antiquity of the species.

Larvae hatch approximately 5 weeks after the female lays her eggs near the high tide mark. The time may vary depending on ambient temperature, and other environmental factors (Jegla and Costlow, 1979). The embryological development of *Limulus* eggs is well described by Kingsley (1892). However, field studies of this phase of the life cycle are limited to observations by Shuster (1958) on nematode, oligochaete and maggot activity in the nests, and Hummon, Fleeger and Hummon (1976) who described the interaction of beach meiofauna with developing eggs.

Newly laid eggs are sticky and occur as tightly clumped balls. When the eggs hatch, the larvae remain in distinct aggregations at depths comparable to those of newly laid eggs. The larvae eventually reach the surface of the sand and emerge into the water column. When larvae emerge from the nest or when they are removed and exposed to water, they exhibit a "swimming frenzy" reminiscent of neonate sea turtles, swimming vigorously and continuously for hours. They are also, like the larvae of many other marine invertebrates, strongly positively phototactic, orienting immediately to any available light source (Thorson, 1964; Rudloe, 1978). Loeb (1893) first mentioned the response of *Limulus* larvae to light, while the development of the swimming behavior has been described by Pearl (1904) as a continuum from embryo to larvae. More recently, French (1977) and French and Doliner (1978) have also described aspects of larval light responses in the laboratory.

While juvenile and adult horseshoe crabs are very similar in morphology, the larval (post hatching) instar is morphologically specialized and unlike all sub-

sequent stages. This larval instar is confronted with the necessity of moving successfully from a nest buried several centimeters beneath the sand surface at a high level of the beach out into the marine environment. Once this transition is achieved, the animal will not re-enter the upper beach environment until it is a sexually active adult. At that time it will be vastly changed, both behaviorally and morphologically, from the larval stage. The timing and mechanisms of release of larval specimens of *Limulus*, as well as the characteristic behavioral patterns of the larvae that contribute to achieving this movement away from the nest and which differentiate it from other phases of the life cycle were the objectives of this study.

MATERIALS AND METHODS

Field studies

Activity of larvae was monitored at Mashes Sands Beach, Wakulla County, Florida from May through November, 1977, June and July, 1978, and in June, 1979. Nests in this area occurred in a distinct zone at the level of extreme high tide and could be easily located by digging at that height on the beach. A series of nests was dug open and the depth from the center of the nest to the surface was measured at periodic intervals ranging from 3 to 7 days between May and November, 1977, for a total of 21 samples. The number of nests measured on each date varied from 10 to 25. Digging was always done within three hours of the daylight high tide.

In addition, the depths of six nests chosen haphazardly each hour were checked over an 11-hr period on the night of full moon in June, 1978. Different nests were measured at each hour, for a total of 66 nests. The nests were dug open by hand and the depth from the center of the larval aggregation to the surface was measured. Nests remained tightly packed after hatching so that accurately locating the center was not difficult. Sampling was also done at the hour of high tide at seven-day intervals thereafter to ascertain nest depths at high tides on the following new moon spring tide and at the following two neap tides. Hourly sampling of six nests was conducted on the night of new moon in June, 1979, using the method described above. Nests were checked from low tide until the hour of high tide, for a total of 48 nests.

Surface plankton tows, each of 4-min duration, were made parallel to the beach at a depth of 1 m for day and night high tides on 128 tides between May and November, 1977, using a coarse mesh net constructed of wire window screening. This mesh retained the macroscopic *Limulus* larvae while passing smaller zoo- and phyto-plankters. The captured larvae were counted and released.

Laboratory analysis of locomotor activity rhythms

Larvae were examined in the laboratory for locomotor activity rhythms using time-lapse photography. An automatically triggered motor-drive 35-mm camera using black and white negative film was used, as was an 8-mm movie camera set for timed exposures. The still camera recorded a frame once every 20 min, whereas the 8-mm camera recorded every 2 min. In all cases the data were grouped into 1-hr intervals.

Activity was recorded under various light regimes. Ambient light (with care taken to insure that no artificial light source was present) with an approximate light regime of fifteen hours of light and nine hours of darkness (LD) and water temperatures ranging between 28° C and 34° C was used to approximate conditions of light and temperature encountered in the upper intertidal zone. Constant light and temperature (LL) and constant darkness and temperature (DD) were also used.

Five hundred larvae were used in each of three DD trials, and 1000 larvae were used in all other trials. These densities did not result in any significant mortality, but, assessed visually, did approximate concentrations observed in the field. Larvae were collected from the field prior to each trial on the nights of full moon, new moon, and at 7-day intervals before and after (the lunar quarters), by digging nests of hatched but unreleased animals. All trials were run until the larvae molted into the morphologically distinct first juvenile instar, usually for 6 to 8 days. Animals held under DD did not molt this rapidly and were tested for 12 days.

An additional four sets of 1000 larvae were tested for 1 week each over 1 lunar month, from full moon to full moon in August, 1978. Trials were initiated on the nights of full moon, full moon plus 7 days, new moon, and new moon plus 7 days, and continued until molting of the larvae as above. Larvae were collected from the field 8 hr prior to the start of each trial, by digging nests of hatched but unreleased animals. An ambient light and temperature regime was employed, and activity was recorded as described above, using time-lapse photography. Lunar variability in the positive response of larvae to a light source was also tested during these trials by exposing the larvae to a constant light gradient during the hours of darkness. This light was provided by an overhead source (Tensor Hi Intensity Lamp #C3812), at a distance of 1 m from the water surface. This illuminated 25% of the test aquarium with white light. The remainder of the aquarium was shaded from the light with an opaque cover, creating a steep gradient of light intensity between the lighted quadrant and the rest of the aquarium.

All trials were conducted in 20-gal glass aquaria with a sand substrate into which animals buried when they were inactive. A closed system of circulation was employed with subsand filtration. Temperatures ranged from 28° to 34° C during the ambient light regime trial and were held at 24° C under constant light and darkness. Salinity was held at 20‰ in all trials.

RESULTS

Field studies

Nest depths in the field showed no consistent change from the beginning to the end of the 8-month breeding season. The mean depth for larvae (13.2 ± 2.9 cm) was not significantly different than that for newly laid and immobile eggs (14.6 ± 1.9 cm) although the standard deviation was somewhat larger ($t = 1.25$, n.s.). However, there was a pronounced short term movement of larval nests upward from a mean depth of 17 cm at low tide to the surface 6 hr later at high tide on the night of full moon (Fig. 1). This was followed by movements of the larval nests back down to depths of approximately 14 cm by the time of the next low tide. Nests could be observed at the surface for 30 min prior to and 30

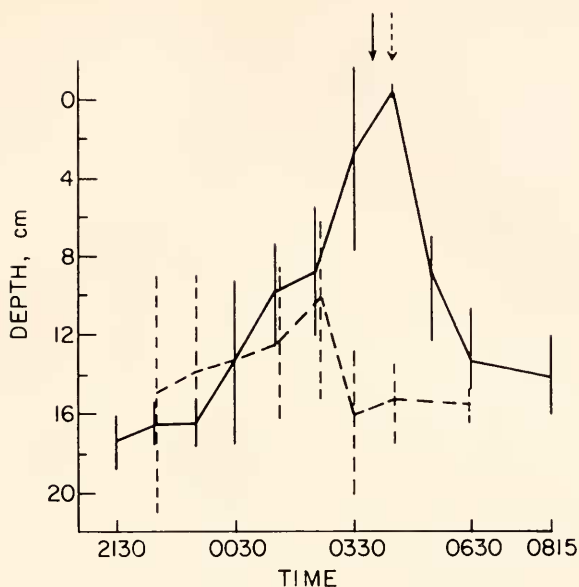


FIGURE 1. Movement of larval nests to and away from the surface of the beach during nights of full moon (solid line) and new moon (dotted line). The arrow indicates the time of high tide in each case. Bars indicate 1 s.d. Movement of nests to the surface on the night of full moon was not repeated on the night of new moon.

min after the high tide. Usually, some 15 to 20 individual larvae become partially exposed at the surface, with the rest of the nest remaining immediately below the surface. Each wave washed away the surface animals and carried them down the beach into the water. Many were cast back up onto the beach and stranded as the tide receded, where they formed drift lines of larvae on the beach. Most of these animals remained at the surface until daylight, after which they disappeared, falling prey to shore birds at dawn. The willet, *Catoptrophorus semipalmatus*, was a major predator. Reburial was probably not of great significance since scattered individuals were only occasionally found beneath the surface. Thus, larvae were seen to move in the field from their original burial depths to the surface and back down again over a 12 hr tidal cycle on the night of full moon. Such pronounced movements to the surface did not occur on the night of new moon. There was no significant difference between mean nest depth at low tide and mean nest depths at high tide on the night of new moon in 1979.

The depths of nests at the hour of high tide on the subsequent neap tide, new moon spring tide and second neap tide of the lunar month in 1978 are presented in Figure 2. Only on the night of the full moon spring tide did larvae come to the surface. The water did not reach the level of the nests at any time other than during spring tides at full and new moon.

The results of the plankton sampling adjacent to the breeding beach on day and night tides throughout the breeding season are provided in Figure 3. The major releases occurred on the night of full moon with two exceptions. And, as with breeding adults (Rudloe 1978), a strong nocturnal activity rhythm is evident.

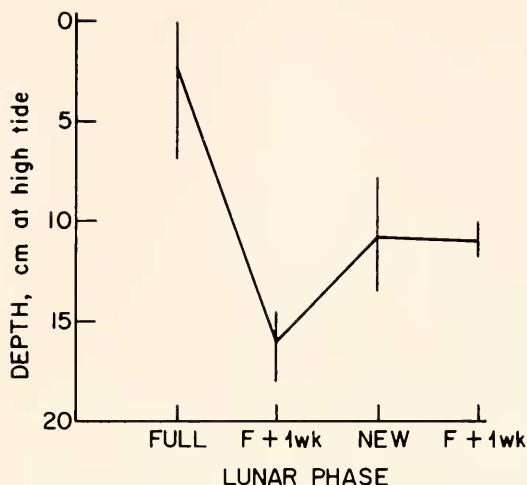


FIGURE 2. Mean depth of larval nests at the hour of high tide at each lunar phase for one month. Bars indicate 1 s.d.

There was also a major release of larvae whenever localized storms with strong onshore winds coincided with high tide, producing an unusually heavy surf. Under these circumstances, larvae were released by day as well as by night. Following a massive storm release in July, the following full moon did not show the usual peak of emergence. The larvae that would have emerged then may have been washed out in the preceding storm. Thus, the occurrence of storm conditions that coincide with a sufficiently high tide are an additional release mechanism.

Laboratory studies

When larvae were maintained under ambient light and temperature regimes, they displayed strong nocturnal activity peaks for the duration of the larval instar (Fig. 4). Larvae remained buried below the surface of the sand substrate during daylight hours. The abruptness with which activity appeared at the same hour each evening and terminated at the same hour each morning suggests that activity might be triggered very precisely by some as yet undescribed factor such as light intensity.

That the nocturnal rhythmicity of behavior might be endogenous in nature is suggested by the results of the trials in which activity was recorded under constant conditions of darkness and temperature. In Figure 5 larval activity is seen to occur during the hours corresponding to normal darkness almost exclusively. It was also noted that none of the larvae used in these trials molted as rapidly to the first juvenile instar as did those maintained under ambient light and temperature conditions.

When maintained under LL conditions and constant temperature, the larvae substrained slight nocturnal activity peaks for the first 3 days (Fig. 6) of the trial, although the amplitude of the variation was considerably reduced, and substantial numbers of larvae remained active during daylight hours. Overall activity was well

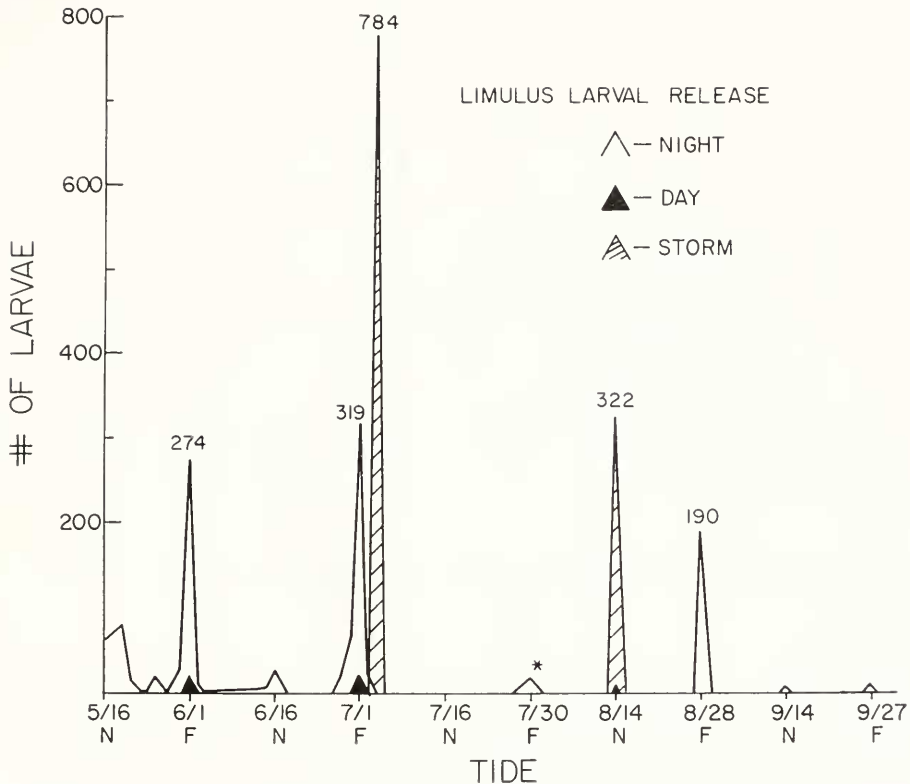


FIGURE 3. Appearance of free-swimming larvae in plankton samples adjacent to a breeding beach throughout a breeding season. F = full moon; N = new moon. Open peaks represent night high tides, closed peaks represent day high tides, and shaded peaks represent storm release. The asterisk denotes the July full moon, when larval release was not significant. This may be due to the extreme release of larvae on the preceding full moon when a massive storm release coincided with the lunar release.

below that under DD and ambient light regimes, however, and reached a level of zero by the fifth day of the trial. When light was removed for 30 min on the fifth night, swimming activity resumed within 5 min of the onset of darkness. After 30 min of darkness, light was restored and activity showed a steady decline throughout the rest of the night. This experiment was terminated thereafter due to the molting of the larvae.

A comparison of levels of activity and light responses of the larvae tested for 7 days each for each week of a lunar month are presented in Table I. Larvae were more active during weeks of spring tides (new and full moon) than on the intervening weeks of neap tides ($t = 5.49$, $P < 0.01$). Differences in levels of activity for the nights of new and full moon in the laboratory were not significant, however.

Although there was no significant difference in over-all first night activity between new and full moon, there was a marked difference in the pattern of activity

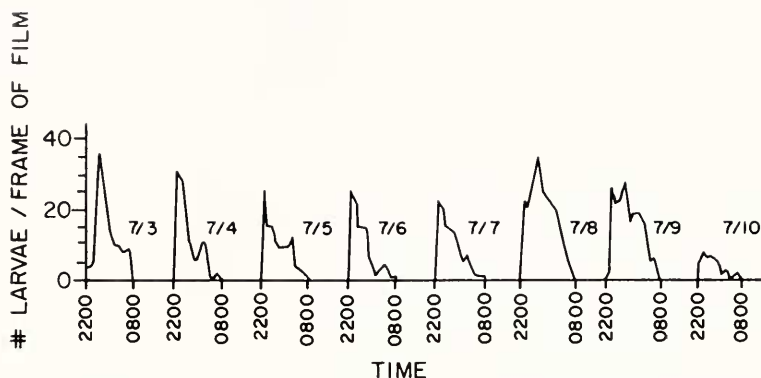


FIGURE 4. Activity patterns of 1000 *Limulus* larvae maintained in the laboratory under conditions of ambient light and temperature. Swimming activity began at 2200 hr each night and terminated at 0800 hr each morning. Larvae remained buried in the sand substrate when not actively swimming. Data is present as number of larvae visible in 1 frame of film at each hour.

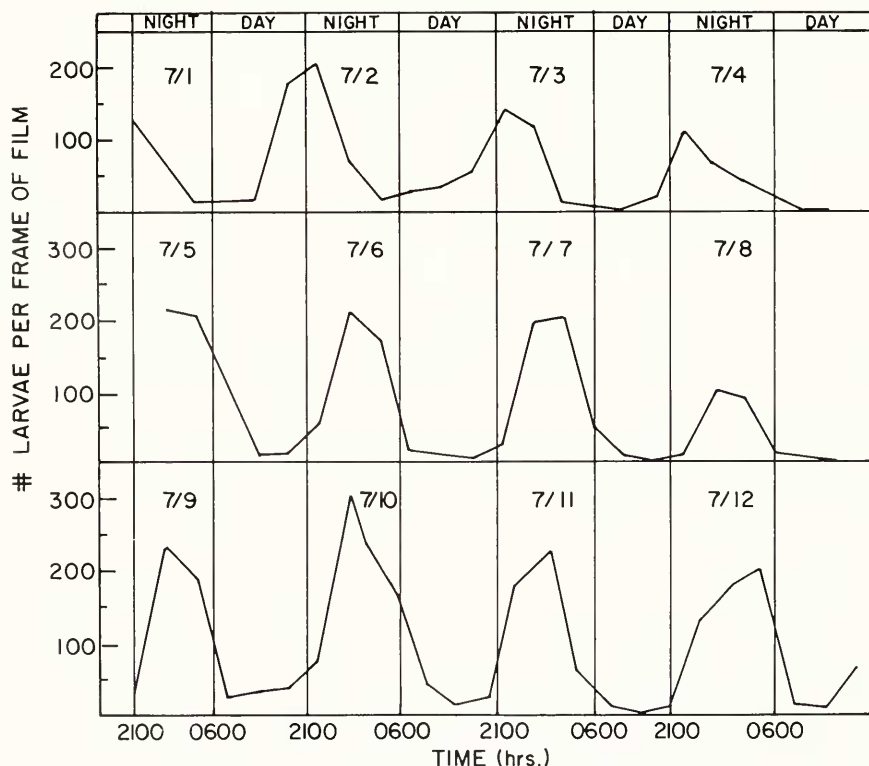


FIGURE 5. Swimming activity of 500 *Limulus* larvae maintained in the laboratory under conditions of constant temperature and darkness. One trial is presented with a duration of 12 days, after which the larvae molted.

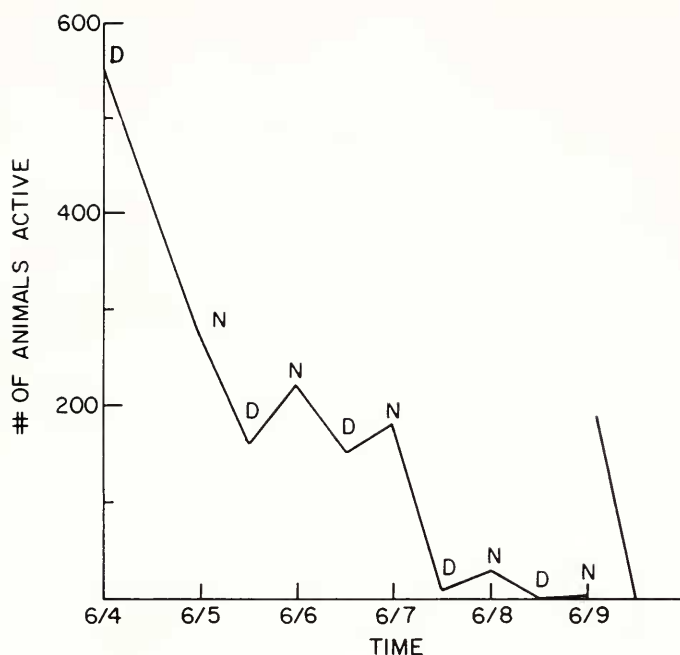


FIGURE 6. Swimming activity of 1000 *Limulus* larvae maintained in the laboratory under conditions of constant temperature and light. Activity terminated on the fifth night but was resumed immediately when light was removed for 5 min at 0100 hr. With restoration of the light, activity again declined. Animals molted during the next 24-hr period and the experiment was terminated. D is daylight at 1200 hr, N is night at 2400 hr. Number of animals swimming was plotted hourly.

during these nights. During the course of the full moon night, activity increased by a factor of 114% between 10 PM and 7 AM while it declined by 29% during the same period during the course of the new moon night. It declined even more steeply, by factors of 79% and 81% on the nights of neap or quarter moon tides. Activity also declined steeply from first to last nights of the session for the full moon (82%) and neap tide (78, 89%) weeks, but declined less sharply from first to last night during the new moon week (30% decline in activity).

Larvae were significantly attracted to light in all lunar weeks (Table I) except that of the new moon. Although larvae were active and swimming on the night of new moon, they showed no significant response to light as measured by concentration of individuals in the lighted quadrant of the aquarium. This lack of responsiveness was maintained throughout the entire week of the new moon trial.

DISCUSSION

The use by *Limulus* of the intertidal sandy beach as a breeding site and the burial of nests well below the surface affords a great deal of protection for developing eggs, and most survive to hatching. The occurrence of larval nests in a distinct zone in the upper intertidal in the northeastern Gulf of Mexico may be related

TABLE I

Lunar variation in larval swimming activity and light responses under laboratory conditions. Four sets of 1,000 larvae, one for each lunar week, were tested. The larvae responded to light at all times other than the week of new moon.

	Lunar phase			
	Full	New	Full - 1 week	Full + 1 week
Mean activity, first night	580	485	329	393
% decline in activity, first night	(increased)	29	79	81
% decline in activity, first to sixth night	82	30	78	89
% responding to light, first night	63	31	78	89
% decline in light response, first night	31	—	38	57
% decline in light response, first to sixth night	32	—	(increased) 113	38
X ² , light response, first to sixth night	49.8	9.44	49.8	64.6
	$P < 0.01$	N.S.	$P < 0.01$	$P < 0.01$
X ² , light response, first night	246	6.52	69.5	131.7
	$P < 0.01$	N.S.	$P < 0.01$	$P < 0.01$

n = 1000 in each trial.

to the small tidal amplitude of approximately 1 m. According to Shuster (personal communication), nests are found in Delaware Bay, where the spring tide range is approximately 2 m, over a much wider band—from the high tide line through 60% of the exposed intertidal. In Cape Cod Bay, where the range is 3 m or more, nests are also widely distributed but tend to be most numerous in the mid tidal area.

In addition to protection, this nesting pattern also creates substantial problems of emergence and dispersal of the young into the aquatic environment. However, *Limulus* larvae are a behaviorally and morphologically specialized phase of the life cycle, that cope well with the problems of nest release. With an initial swimming frenzy and positive phototaxis, and with lunar and circadian activity rhythms that synchronize activity with water levels on the beach, the larvae are well adapted for emergence from the nests and dispersal. After this transition is achieved, larvae molt into the substantially different morphology and behavior patterns characteristic of the benthic portion of the life cycle.

Although further work is required to be conclusive, the nocturnal swimming of *Limulus* larvae under DD conditions suggests an endogenous rhythm. The peaks of activity appear to occur progressively later during the night as the experiment progresses, and these peaks are approximately associated with the time of low tide in the field. Whether this is in fact reflective of an advancing endogenous tidal cycle or is a shifting of the circadian rhythm is not established at this time. The observed suppression of activity under LL is characteristic of both aquatic and terrestrial nocturnal species (Aschoff, 1960).

Semi-lunar reproductive rhythms are well known among intertidal crustaceans, snails, and bivalves (Gifford, 1962; Warner, 1967; Russell-Hunter, Apley and

Hunter, 1972; Wheeler, 1979). The field emergence of larvae on the full moon and the absence of larval release on the new moon therefore poses an intriguing question. Is it reflective of an endogenous lunar rhythm, or is moonlight a key stimulus in orienting the movement of larvae to the surface and into the water column?

Based on the activity of larvae in the laboratory, there appears to be a lunar rhythm of responsiveness to light that is somewhat different from the lunar locomotory rhythms observed. While larvae were active at both full and new moons (*i.e.* on spring tides), they were responsive to light only at full moon. This is consistent with the lack of field larval release during the spring tides of the new moon.

Furthermore, neurophysiological sensitivity to light several hundred times dimmer than moonlight has been recorded for the *Limulus* median ocellus by Lall and Chapman (1973) who obtained responses to levels of illumination as low as $1.4 \times 10^{-10} \mu\text{W}/(\text{cm}^2 \cdot \text{nm})$ at 360 NM. Moonlight intensity of $10^{-3} \mu\text{W}/(\text{cm}^2 \cdot \text{nm})$ has been reported by Kampa (1970). Therefore moonlight is well within the sensitivity range of adult specimens of *Limulus*.

A model to explain the observed pattern of larval release in the field is presented. It is based on the comparison of field release with the activity and light response patterns seen in the laboratory. The following factors are apparently of significance: an endogenous readiness to respond to a dim light source that peaks at the time of full moon and is minimal at the time of new moon; the availability of an appropriate light source in the form of a full moon; a water level reaching the nests only during periods of spring tides; and the suppression of activity by the bright light of day that overrides afternoon spring high tides. This pattern of larval release is supplemented by occasional mass releases caused by storms that coincide with high tides at times other than full moon.

This model seems to be most consistent with the data presently available. In particular, the full/new moon differences in response to light in the laboratory correspond to the lack of field release at new moon. However, whether moonlight or the lack of it is detectable to larvae buried several centimeters in the sand prior to high tide has not been determined. If it is not detectable at the depth of the nests, then some other factor may be instrumental in initiating movement toward the surface.

If inactive larvae are removed from a nest and placed in water, swimming activity is immediately initiated. Increasing interstitial moisture associated with the rising tide might conceivably initiate activity, for instance, with moonlight becoming the dominant stimulus only as the animals approach the surface. The apparent partial movement of larvae toward the surface on the night of new moon in June, 1979, suggests the existence of some stimulus in addition to moonlight. The identification of that stimulus, should it exist, as well as testing of the other components of the proposed model, awaits additional research.

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SUMMARY

The horseshoe crab *Limulus polyphemus* lays its eggs on sandy beaches at the level of the highest high tide in the northeastern Gulf of Mexico, buried approximately 18 cm below the surface. When they hatch, the larvae must move from the buried nest site into the marine environment.

In the field, nests of larvae move to the sand surface and emerge at the spring high tide on the night of full moon. They may also be released by heavy surf associated with storms. No release occurs on the spring high tides associated with new moon.

In the laboratory, larvae are seen to be nocturnally active, both under ambient and DD photoperiods. Activity peaks at times of full and new moons, and larvae are positively phototactic at all lunar phases except new moon.

A model to account for observed field behavior in light of laboratory activity and light responses is presented.

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